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Histology of Dichromatic and Seasonal Color Change of the Cranial Region of Callagur borneoensis

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HISTOLOGY of DICHROMATIC and SEASONAL COLOR CHANGE of the CRANIAL REGION of CALLAGUR BORNEOENSIS

(TITLE)

BY

Kevin William Matson

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HISTOLOGY OF DICHROMATIC AND SEASONAL COLOR CHANGE OF THE CRANIAL REGION OF *CALLAGUR BORNEOENSIS*

by

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Life Sciences Division

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ABSTRACT

Male Callagur borneoensis exihibit sexual dichromatism and seasonal coloration which is rare among turtles. In the breeding season male's heads are colored white with a scarlet stripe from the occiput to the tip of the snout. After the breeding season they change to drab charcoal-gray to black with a dull orange-yellow stripe. Females and juveniles are a drab brown throughout the year.

This study was performed to determine the mechanisms of this color change at the histological level. Histological preparation of the head revealed a significant increase in vascular tissue just below the epidermis of the stripe area with increased red color.

The white color of the side of the head was attributed to no epidermal melanosomes and thickening of the epidermis. Dermal melanin was found to play a lesser role in determining the darkness of the skin. Dermal melanophores appeared to have a cycle during the color change. In dark phase I, they were the most prominant donating melanosomes to the epidermis. In dark phase II they were the least prominant appearing to donate melanosomes to the epidermis and blood vessels along which they were aggregated. The melanophores of the light phase were slightly more prominent and were not donating any melanosome to the epidermis. The intermediate had more prominent melanophores that were donating melanosomes to the epidermis.

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ACKNOWLEDGMENT

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INTRODUCTION

Callagur borneoensis is a predominately aquatic emydine turtle inhabiting the rivers of Malaysia, Borneo, and Sumatra. Mature female Callagur have a carapace length range of 400 - 500 mm. Males range from 280 - 400 mm. (Moll, 1979 - personal communication).

Sexual dichromatism is rare in turtles and seasonal coloration is rarer still. Callagur borneoensis exhibits both. In the breeding season males heads are colored white with a scarlet stripe from the occiput to the tip of the snout. After the breeding season they change to drab charcoal gray to black with a dull orange-yellow stripe. Females and juveniles are a drab brown color throughout the year.

This study was undertaken in an attempt to explain the mechanisms of color change of the occipital and stripe regions of the head at the histological level.

There is little published literature on the soft parts of the turtle integument at the histological level. Structure of the epidermis and cysts were noted by Lange (1931), Stolk (1956), and Spearman (1969). Specialized organs of the epidermis were noted by Smith and Brown (1946), and Winokur and Legler (1974, 1975). Only Auffenberg (1964), and Smith et. al. (1949) published material on the color change in turtles at the histological level.

MATERIALS AND METHODS

Six mature Callagur borneoensis (males EOM2307, light phase; EOM2255 and EOM2222, intermediate phase; EOM2335 and EOM2306, dark headed phase; a female EOM2390) collected from the Perak River, Perak West Malaysia in 1975 by Dr. Edward O. Moll were used for histological examination. The only liver available was from an intermediate phase (EOM2222) whose head and skin had previously been sent to another institution. These had been killed by injecting 70% alcohol into the intervertebral space behind the last cervical vertebra. The skins and heads were fixed in 10% formalin and stored in 70% alcohol. The liver was preserved and fixed in 10% formalin.

Tissue blocks were taken from the right side of the head in the occipital region, from the stripe near the tip of the snout, from the oral cavity slightly behind the internal nares opening, and from the liver. These were dehydrated, cleared, embedded in paraffin, and sectioned at 7-9 u on a rotary microtone. Stains used were Harris Hematoxylin and Eosin, Nile Blue, and 1:3 dilution of Anilin Blue Collagen Stain (Conn etal, 1963).

Additional blocks from the left side were removed and trimmed with a razor blade to be examined by scanning electron microscope. These were prepared by Dr. William James by mounting on studs, critical point drying, and gold sputter coating. The tissue blocks were observed at 15KV and pictured in a JSM-U3 JEOL* Scanning electron miscroscope.

*Japanese Electron Optical Laboratories

Determination of per cent area occupied by vascular material was accomplished on the Anilin Blue slides as follows; measurements were made at 430 magnification using Baush and Lomb Micrometer Disc 31-16 13 in the right ocular of the scope. A section was brought into the edge of the field of view and horizontal control was given 1/8 of a turn (with eyes away from the scope). The horizontal control was not touched after this. Using the vertical control, the section was then positioned so that the top of the grid was just below the basal cell of the stratum germinativum. The ocular grid was rotated to get the best alignment of the top line of the grid with the dermal-epidermal interface. The per cent of each square occupied by vascular material was estimated and recorded. The grid was again advanced 1/8 of a turn and the process repeated. 100 such counts were made.

The same procedure was used to estimate the per cent area occupied by melanin. However, the per cent of melanin was so low that this method of approximating percentage of each square had to be refined. The center square of the grid is subdivided into twenty-five squares, each occupying 4% of the larger square. By actually counting the number of small squares filled or partially filled with melanin I was able to calculate the percentage of melanin for the center square. By mentally calculating the number of these 4% squares the melanin each larger square would fill, I was able to estimate to my satisfaction the percentage of melanin in each larger square. The means of per cent area occupied by melanin and vascular material were tested for signifi-

cant difference using the Student's t distribution at the 95% confidence level.

The total amount of light reflected from the typanum of the head of the preserved specimens was determined by stop down metering by Charles Miller of the Department of Physics, Eastern Illinois University, using a Nikkormat FTN camera with M-2 extender ring and a 55mm F 3.5 Micro Nikkor Lens allowing a 1 cm field of view. The aperature was adjusted with the camera set for film speed of ASA400 and 1/8 second shutter speed and the f stop values were recorded. In addition, f values for reference points were determined on Kodak standard grey card which reflects 18% of incident light and a standard white card reflecting 90% of incident light registering 58 ft/candle at the specimen.

Herein the term Melanophore will refer to a cell containing melanin and melanosome will refer to the melanin containing organelles of a melanophore which may be donated to other structures.

OBSERVATIONS

General Description

The head of the female is solid drab brown to yellow-brown. White phase male heads are white and puffy with a scarlet stripe extending from the occiput to the tip of the snout. The head of the intermediates are grey with a less intense red stripe. Dark phase males are much darker being black to charcoal in color (Moll, 1979, personal communication).

Dr. Edward Moll and I attempted to visually rank the preserved male specimens in order of darkness. Both ranked in the same order i.e. EOM2307, EOM2255, EOM2306, EOM2335, with EOM2335 being the darkest. Step down metering was used to confirm the visual ranking respectively - f value of 5.6, 4.0, 2.8, and 2.8 (Figure 1). As a result EOM2307 was classified as light, EOM2255 as intermediate, and EOM2306 and EOM2335 as dark phase. EOM2335 appeared slightly darker than EOM2306. EOM2335 had more color in the stripe, this being yellow-orange.

Winokur (1973) recognized three epidermal layers of turtles - the superficial stratum corneum, intermediate stratum granulosum, and the basal stratum germinativum. Lange (1931) and Winokur (1973) recognize two dermal layers - superficial stratum laxum composed of loose collagen bundles running at all angles, and a deep stratum compactum composed of highly organized dense collagen fiber running perpendicular to each other.

Epidermis

Microscopic examination of the epidermis revealed differences in the occipital region of the heads of specimens. The thickness and make up of the three epidermal layers differed with the phase and gender (Table 6). In the light phase of the male the epidermis was much thicker due to proliferation of the stratum corneum and stratum granulosum (Figure 3). The stratum corneum was extremely thick and granular (Figure 4). Cells of the granulosum were very large filled with karahylin granules and had prominent intercellular bridges (Figure 6). A network of interconnecting channels (presumably fluid filled) projected .61 to .73 mm (Table 5) upward from the dermis into the granulosum dividing it into irregular shaped polyhedrons .38 - .45 mm in diameter (Figure 3, 5 and 7). The granulosum cells directly above and along the channels were smaller and irregular creating a distinct pattern. Dermal papillae containing collagen fibers, blood vessels, and melanophores extend into these channels. The upper portion of the channels were bulbous and lined with what appeared to be endothelium. Frequently the channels transected a polyhedron (Figure 7). The tops of the polyhedrons were domed. The entire surface of the occipital area was covered with these shallow domes. An electron miscroscope scan revealed these were covered with small proturberances (Figure 8) presumed to be the small plugs with pits above them produced at the end of the channels seen in the longitudinal section in Figure 3.

Transverse sections pictured this small plug as an amorphous mass surrounded by concentric horny layers similar to the cysts described by Stolk (1956) and Spearman (1969) (Figure 9). Often the amorphous mass was missing, persumably shed to the surface, leaving only a concentric horny ring (Figure 7). Other structures observed in transverse section were hollow or fluid filled tubes lined with what appeared to be an endothelium lining (Figure 9). These were presumed to be extensions of channels that had been pinched from the main channel.

In the intermediate phase, EOM2255 the epidermis in the occipital region was much thinner. Accurate readings of the stratum corneum were not possible becasue this layer tended to slough off during sectioning. The stratum granulosum was thinner due to both a reduction in cell size and reduced number of cells in the tissue (Figure 10).

The epidermis of the dark phase males was even thinner. In EOM2335 all three layers could be seen (Figure 11) but in EOM2306 the stratum granulosum was indistinct (Figure 12, 13). In both turtles the stratum corneum was much more compact and was not granular. The dermal channel, although much reduced, still formed ridges around the polyhedrons but the polyhedrons were so reduced that they were not domed out cupped into the ridge formed by the channels (Figure 14) making the skin appear to be covered with craters. Often small protuberances were observed on the rim of these craters (Figure 15).

The epidermis of the female EOM2390 was similar to that of the dark phase males, except that it lacked dermal channels. All layers

were thin and distinct (Figure 16). When viewed from above, the surface of the skin appeared relatively smooth.

Pigment

The amount and distribution of pigment changed as the thickness of the epidermis changed (Table 2 and 3). The light phase (EOM2307) had no pigment in the epidermis and melanophores were light brown. They apeared to aggregate along blood vessels (Figure 17). The same phenomenon was noted by Smith and his colleagues (1949) in Trionyx spiniferus. Very denteric melanophores were observed mostly within a strip .0525 mm below the stratum germinativum. The epidermal pigmentation of the intermediate (EOM2255) was variable. Some areas had a few melanosomes in the basal lamella - other areas none. Denteric, dark brown melanophores were most prominent in the area .0525 mm below stratum germinativum (Figure 18).

Dark male (EOM2335) had the most epidermal pigmentation, but had only occasional melanophores within .0525 mm of the stratum germinativum. Of the melanophores that were present below the .0525 mm layer, nearly all occurred along the blood vessels (Figure 19).

The other dark male (EOM2306) had slightly less epidermal pigment than (EOM2335) but the dermal melanophores were much more prominent than in any other male.

The pigmentation of the female (EOM2390) was similar to the dark male (EOM2335). The melanophores of the female were large and punctate

(Figure 20).

Per cent of area of .175 mm under stratum germinativum occupied by melanophores are presented in Table 2. At the 95% confidence level, dark phase EOM2335 was shown to have significantly less dermal pigment than light phase EOM2307. Dark phase EOM2306 had significantly more dermal pigment than either EOM2255 or EOM2307.

Red Stripe

Sections of the red stripe stained with Anilin Blue Collagen Stain were compared. The stratum germinativum was one cell deep having especially prominent cytoplasmic extensions into the dermis, (Figure 21). These were referred to as "root processes" by Andrew (1959) and Winokur (1973). Relative proportions of the different cell layers are summarized for each individual in Table 7.

The stratum corneum comprised the greatest portion of the epidermis in all phases. This clear, compact area was thickest in the dark phase EOM2306 with 52 cell layers as compared to 37 cell layers in the light phase EOM2307 and 28 in intermediate phase EOM2255 and 48 in EOM2335. The stratum granulosum was comparatively thin but was again thickest in the dark phase EOM2306.

The stratum laxum of the dermis (just below the stratum germinativum) was highly vascularized in the light phase (Figure 22). The averge area occupied by vascular tissue (within 0.175 mm of the stratum germinativum) significantly increase from 0.47% in the dark

phase (EOM2306) to 28.24% in the light phase (Figures 22, 23, 24, 25). The dark phase (EOM2306) had fewer, smaller blood vessels (Figure 25). The blood vessels of the intermediate phase (EOM2255) were numerous, but the diameters were generally smaller than those in the light phase (EOM2307).

Oral Cavity

The interior of the oral cavity in all forms was white and was covered by stratified epithelium. The underlying dermis was composed of loosely arranged collagen fibers running parallel.

Liver

The liver of the intermeidate phase EOM2222 had round to oval nests of cells full of melanin similar to those described for Trionyx spiniferus by Smith and his colleagues, (1949). The diameter of these nests reached .16 mm (Figure 26). Melanin was also observed in the blood sinusoids of the liver.

Spermatogenic Stage

Testes of six males were observed and ranked according to spermatogenic stage by Dr. Edward Moll (1979, personal communication) his results are as follows; Dark phase I EOM2306 - Spermiogenesis (tailed sperm beginning to cluster at Sertoli cells), Dark phase II EOM2335 - Spermatocytogenisis (spermatocytes are predominant cell types, few

spermatids and no tailed sperm persent), Intermediate EOM2222 Gonial proliferation (spermatogonia predominate only a few scattered spermatocytes present), Intermediate EOM2255 - Gonial proliferation (Spermatogonia predominate no spermatocytes evident), Light phase EOM2307 - Spermiation (Mature sperm fill the lumina of all tubules. spermatocytes and spermatids nearly exhausted).

DISCUSSION

White head coloration in Callagur appears structural in nature. Structural colors result from interference, defraction, and tyndall scattering of light by colloidal or fine crystaline materials which includes keratin (Mason, 1923, 1926; Bagnara and Hadley, 1973). Fox (1976) reported that white colors in the epidermis of certain reptiles are due to the structural effects of keratin.

In the white headed Callagur the thickened epidermis separates the pigmented area of the dermis from the surface to the extent that the melanin has little effect on the surface color. Rather keratinized stratum corneum and stratum granulosum reflect and refract the light providing the white coloration. The effects of melanin are further reduced in the white phase as the melanin granules (melanosomes) often present in the basal layers of the epidemis in other phases are absent here.

Since the darkest individual EOM2335 had less dermal pigmentation than the light phase (EOM2307) dermal pigmentation would seem to have less effect than the melanosomes in the epidermis; on the color of the males other than that they donate melanosomes to the epidermis. The darkest coloration appears to result from epidermal melanosomes.

The red color of the head stripe is seemingly brought about by increased blood flow to the area. Fox (1976) and Leeson and Leeson (1976) cited hemoglobin a a source of red color. Auffenberg (1964)

mentioned area color change in turtles due to increased vascularization. The yellow-orange color of the darker phase is possibly due to the light being reflected and refracted by the stratum corneum and the underlying dermis. Since the stratum corneum is organized differently in this area than in other areas, it is possible that it would produce a different color effect. However, the color could be due to a carotinoid pigment. Since carotinoid pigments are very soluble in alcohol, most would have been removed during the preservation process so no attempt was made to find them in the preserved tissue.

Based on the four male specimens melanogenesis reaches its peak in the early dark stage when the epidermis is thin and pigmented and the dermis well supplied with denteric melanophores. At this stage most of the red coloring has been lost from the red stripe due to the loss of blood sinusoids. This is the stage of EOM2306. Undoubtedly when gonial proliferation begins, melanogenesis stops and process of losing the melanin from the dermis and epidermis begin. At the same time the blood sinusoids start to increase bringing red color to red stripe area. The fact that the dermis of EOM2335 had lost most of its melanophores while the epidermis was stil well supplied with melanosomes would indicate a rapid removal of melanin from the dermis, possibly by absorption into the blood and slow removal in the epidermis possibly by shedding with the stratum corneum. The evidence of melanin in the liver suggests that possibly melanin is transported there to be disposed.

When full breeding condition is reached the blood sinusoids below the stripe have their greatest capacity and the epidermis of the occiputal area is at its thickest. While still in breeding condition melanogenesis starts again as evidenced by EOM2307 which had an increased number of melanophores in the dermis when compared to EOM2335. As breeding condition recedes the transfer of melanin to the thinner epidermis darkens the appearance of the skin and the reduction of the blood sinusoids depletes the red color from the red stripe area. EOM2255 was presumably in mid state from light to dark since he contained more denteric melanophores than either the late dark EOM2335 or the light phase EOM2307.

This scheme coincides with Dr. Edward Moll's arrangement of the spermatogenic cycle with the exception of dark phase II EOM2335 which would rank it between the intermediate EOM2255 and the dark phase I EOM2306.

CONCLUSION

The red stripe can be attributed to increased vascularization of the stipe area.

Epidermal melanin and thickness of the epidermis are the most important factors in dermining the darkness of the side of the head, since Dark phase II was the darkest yet had the least dermal melanin. The Light phase is result of the total lack of epidermal pigment and the extreme thickness of the epidermis which reflects the incident light.

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TABLE 1 Plastron and Carapace length in mm for specimens in this study

	Males				Female
	Light 2307	Inter. 2255	DarkII 2335	DarkI 2306	2390
Plastron	305	318	310	295	440
Carapace	348	358	259	334	482

TABLE 2 Per cent of stratum laxum (0.176 mm under stratum germinativum) occupied by melanophores in skin of male occipital region

	Light 2307	Inter. 2255	DarkII 2335	DarkI 2306
$\bar{x}$	5.81	6.28	2.42	10.04
SD	1.27	1.44	1.22	3.60
Range	3.04-9.12	3.48-12.54	0.16-6.66	4.16-19.70
N	100	100	100	100

TABLE 3 Amount of melanin in epidermis

	Males				Female
	Light 2307	Inter. 2255	DarkII 2335	DarkI 2306	2390
	-	$\pm$	++	+	++

\- = no melanosomes evident in the epidermis
$\pm$ = melanosomes evident in some parts of the basal lamella
$\overline{+}$ = melanosomes evident in all of the basal lamella
++ = many melanosomes evident in all the basal lamella

TABLE 4 Per cent of stratum laxum (.017 mm stratum germinativum) occupied by blood vessels in the red stripe region

	Light 2307	Inter. 2255	DarkII 2335	DarkI 2306
$\bar{x}$	28.24	5.76	10.99	0.47
SD	8.79	3.11	5.00	0.92
Range	7.00-46.00	1.20-14.00	3.00-27.00	0.00-4.00
N	100	100	100	100

TABLE 5 Length in mm that channels extend into epidermis in each color phase

Light 2307	Inter. 2255	DarkII 2335	DarkI 2306
.61-.73	.032-.043	.020	.020-.053

TABLE 6 Comparative depths (mm) of epidermal layers in female and four males of the lateral side of the head representing three color phases N = 100 for each characteristic examined.

	Males				Female
	Light 2307	Inter. 2255	DarkII 2335	DarkI 2306	2390
STRATUM CORNEUM					
#layers	27	8*	13	6	2-4
depth of layer $\bar{x}$	.11		.021	.035	.023
SD	.038		.012	.027	.014
Range	.020-.19		.037-.0049	.012-.057	.0025-.029
cell diameter	.006	ND	.001	.007	.006
STRATUM GRANULOSUM					
#layers	44-48	5-10	2-3	0-2	2-3
depth of layer $\bar{x}$	.75	.13	.010	.011	.015
SD	.13	.11	.0085	.011	.0058
Range	.58-1.02	.029-.17	.025-.049	0-.049	.0025-.027
cell diameter	.026-.019	.012	.006	.001	.008
STRATUM GERMINATIVUM					
#layers	2	2	1-2	2	1-2
depth of layer $\bar{x}$	.033	.57	.018	.029	.023
SD	.014	.022	.0054	.012	.0086
Range	.010-.097	.019-.068	.007-.030	.012-.074	.0025-.040
cell diameter	.048	.012	.031	.032	.12

* = possibly more layers; stratum corneum is lost during sectioning process

TABLE 7 Comparative depths (mm) of epidermal layers in female and four males of the striped area of the head representing three color phases N = 100 for each characteristic examined.

	Males			
	Light 2307	Inter. 2255	DarkII 2335	DarkI 2306
STRATUM CORNEUM				
#layers	37	28	48	52
depth of layer $\bar{x}$	.16	.20	.16	.21
SD	.021	.033	.027	.0093
Range	.11-.19	.14-.28	.064-.22	.16-.29
STRATUM GRANULOSUM				
#layers	3	6	6	5-7
depth of layer $\bar{x}$	.016	.05	.023	.26
SD	.0053	.018	.0088	.013
Range	.007-.037	.017-.092	.005-.042	.007-.049
STRATUM GERMINATIVUM				
#layers	1	1	1	1
depth of layer $\bar{x}$	.027	.030	.032	.024
SD	.0082	.026	.10	.0088
Range	.001-.050	.005-.054	.007-.049	.007-.064

FIGURES

1) Per cent of incident light reflected from surface of the head in the male study specimens.

2) Proposed cycle of the skin of Callugar borneoenis

3) Occipital region of 2307, longitudinal section of the epidermis showing (PL) Plug, (Ch) Channel, (Po) Polyhedron. X100.

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9) Occipital region of 2307, transverse section of epidermis showing (Ac) Amorphous center (Hc) Hollow channel. X430.

10) Occipital region of 2255, longitudinal section of epidermis showing (Ch) Channel, (Sg) Stratum granulosum. X430.

11) Occipital region of 2335, longitudinal section of epidermis showing (Sc) Stratum corneum, (Sg) Stratum granulosum, (Sr) Stratum germinativum - arrows are areas of high melanosome concentration. X430.

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17) Occipital region of 2307 Dermis showing (Bv) Blood vessels (M) Melanophores. X430

18) Occipital region of 2255 showing (Ep) Epidermis, (Dm) Denteric Melanophores. X430

19) Occipital region of 2335 longitudinal section showing (M) Melanophores, (Sc) Stratum corneum, (Sg) Stratum granulosum, (Sr) Stratum germinativum. X430

20) Occipital region of 2390 female longitudinal section showing (Ms) Melanosomes. X430

21) Stripe area of 2335 longitudinal section of the red stripe showing (Bv) Blood vessel, (Rp) Root processes, (Sc) Stratum corneum, (Sg) Stratum granulosum, (Sr) Stratum germinativium. X430

22) Stripe area of 2307 longitudinal section showing (Bv) Blood vessel. X100

23) Stripe area of 2255 longitudinal section. X100

24) Stripe area of 2335 longitudinal section. X100

25) Stripe area of 2306 longitudinal section. X100

26) Stripe area of 2222 liver showing (Mn) Melanin nest cells. X430

Figure 1 Per cent of incident light reflected from surface of the head in the male study specimens

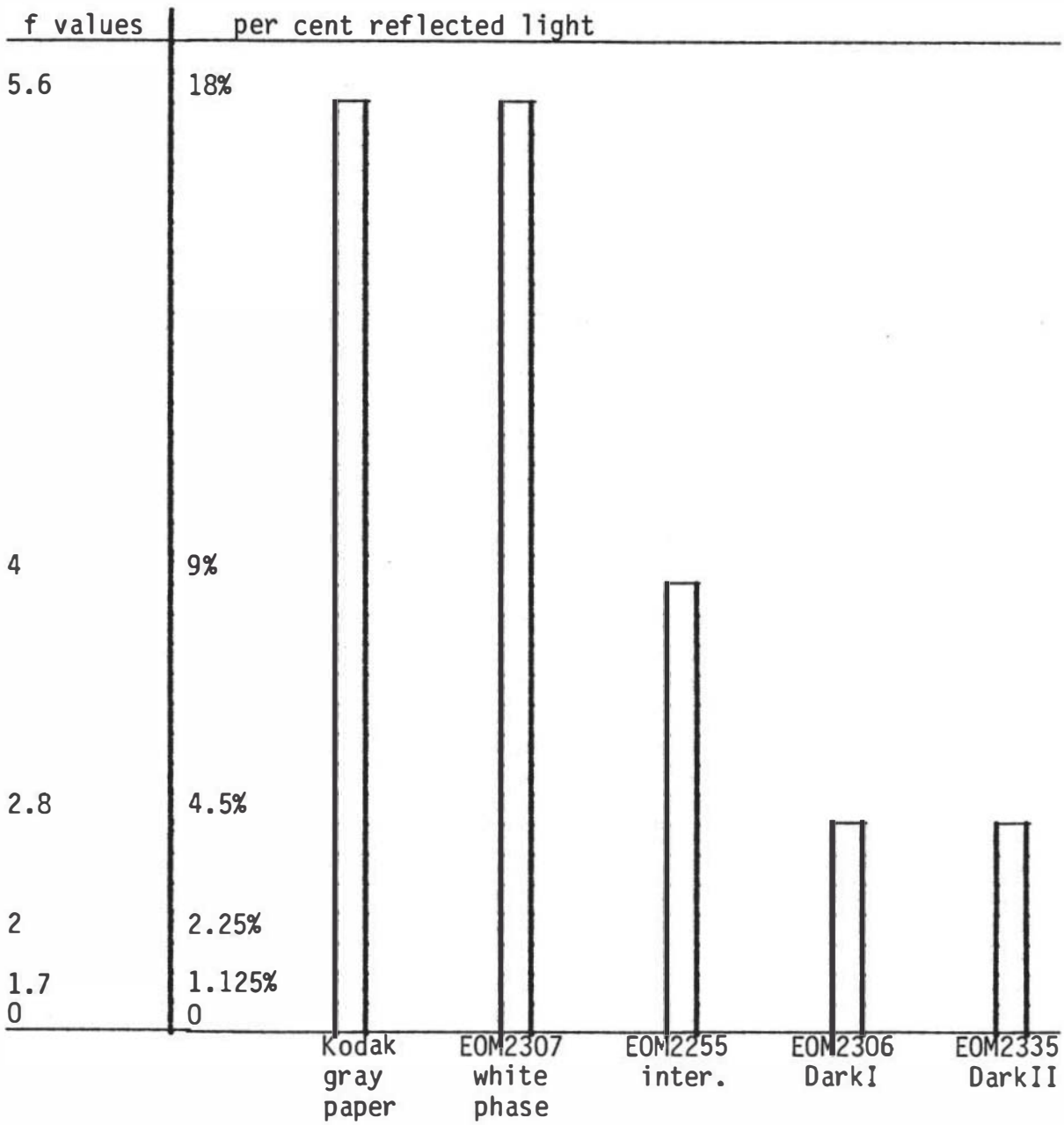
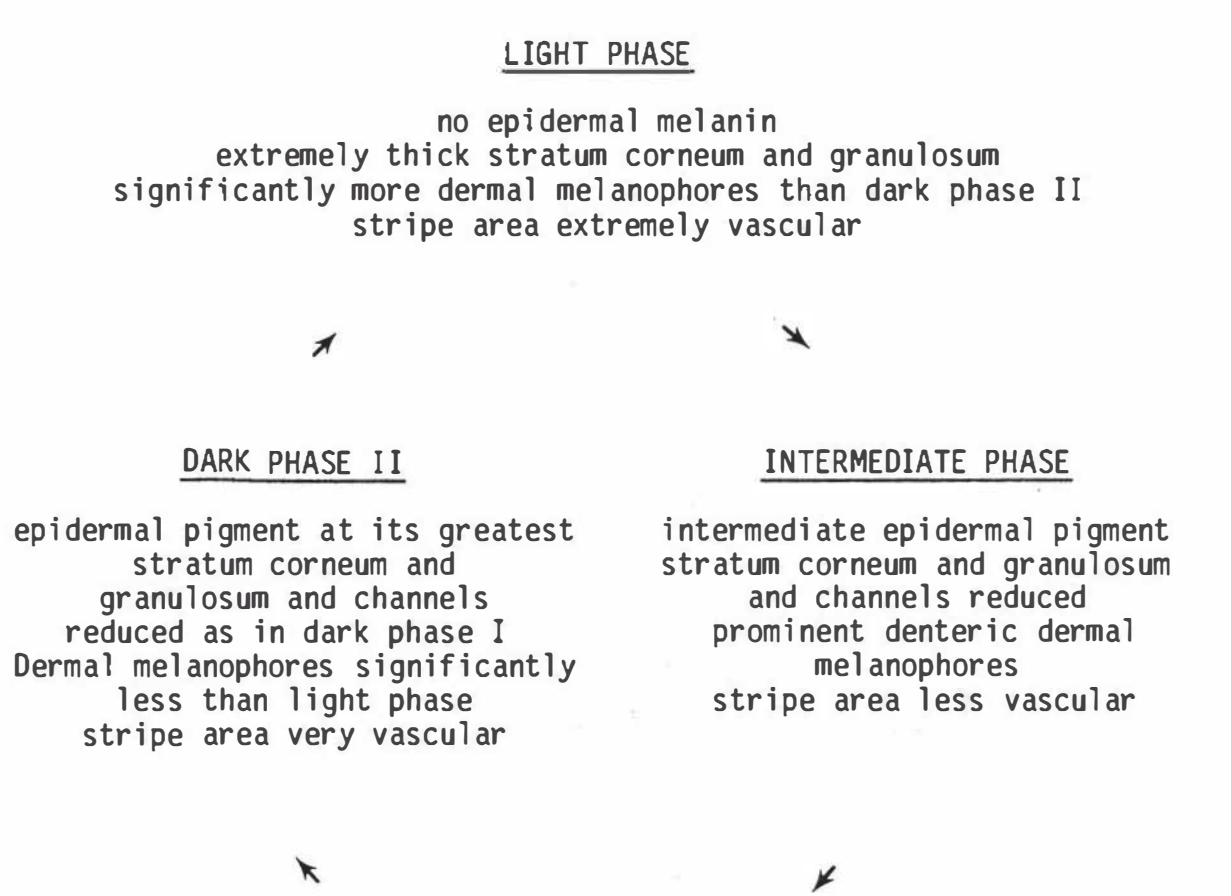
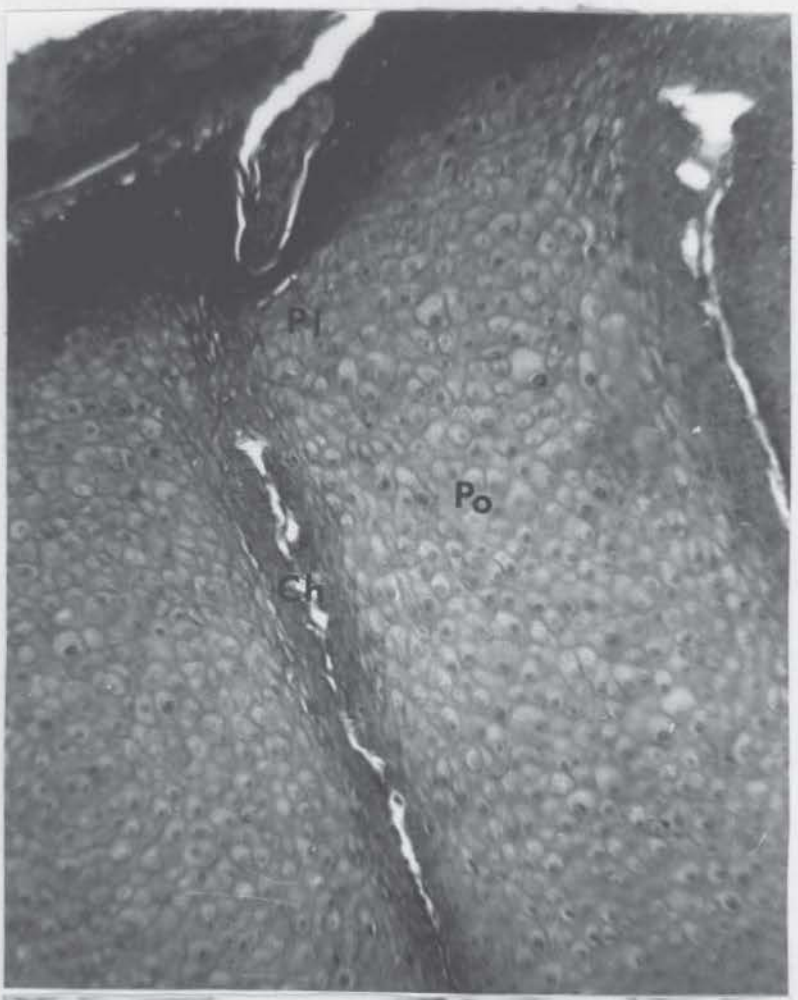


Figure 2 Proposed cycle of the skin of Callagur borneoenisis





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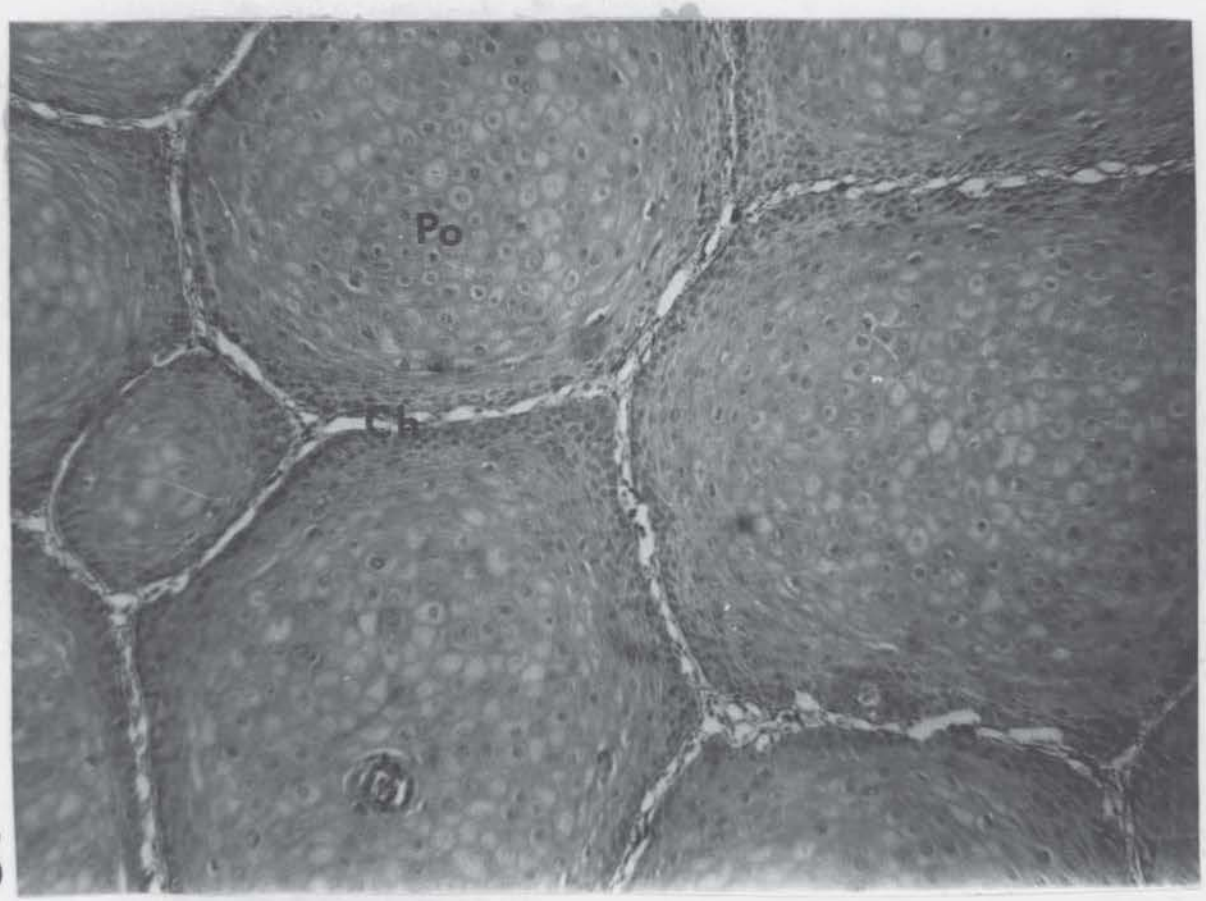

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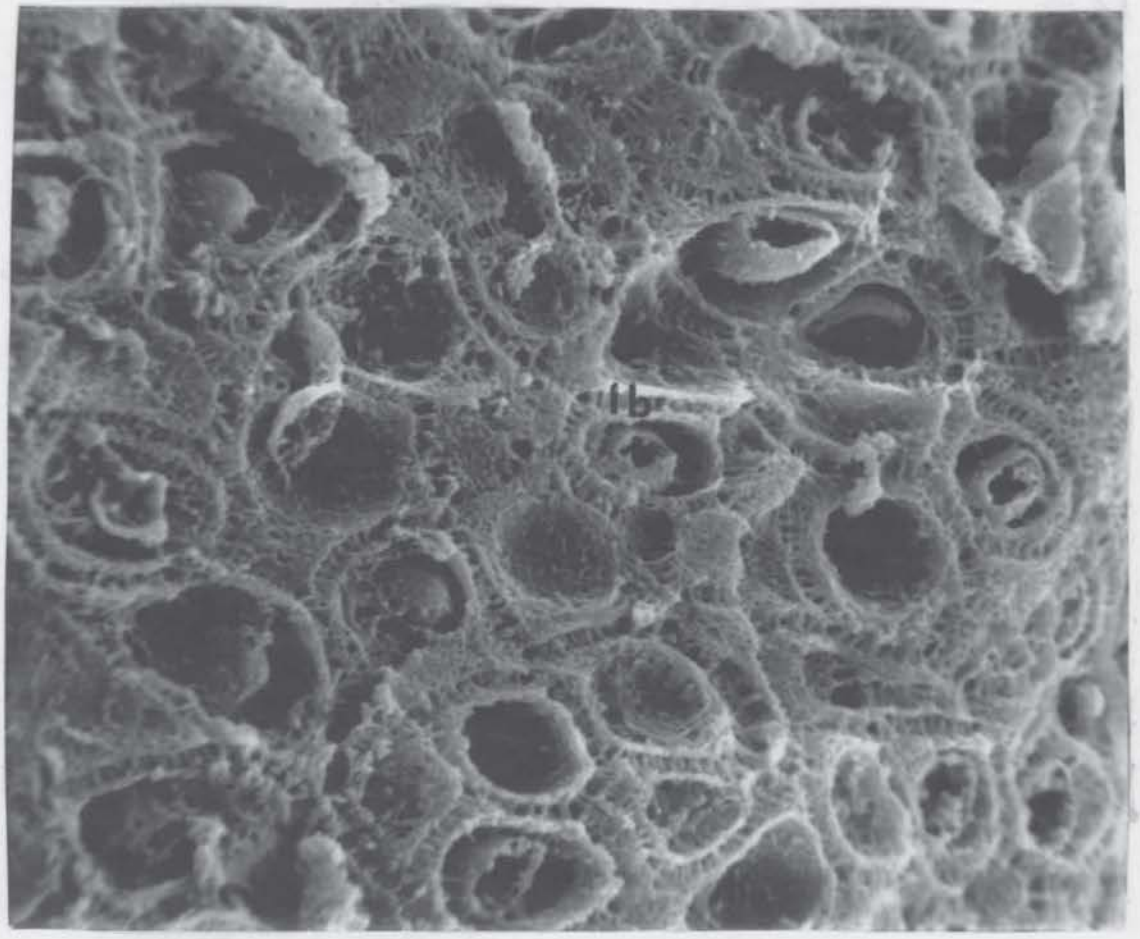

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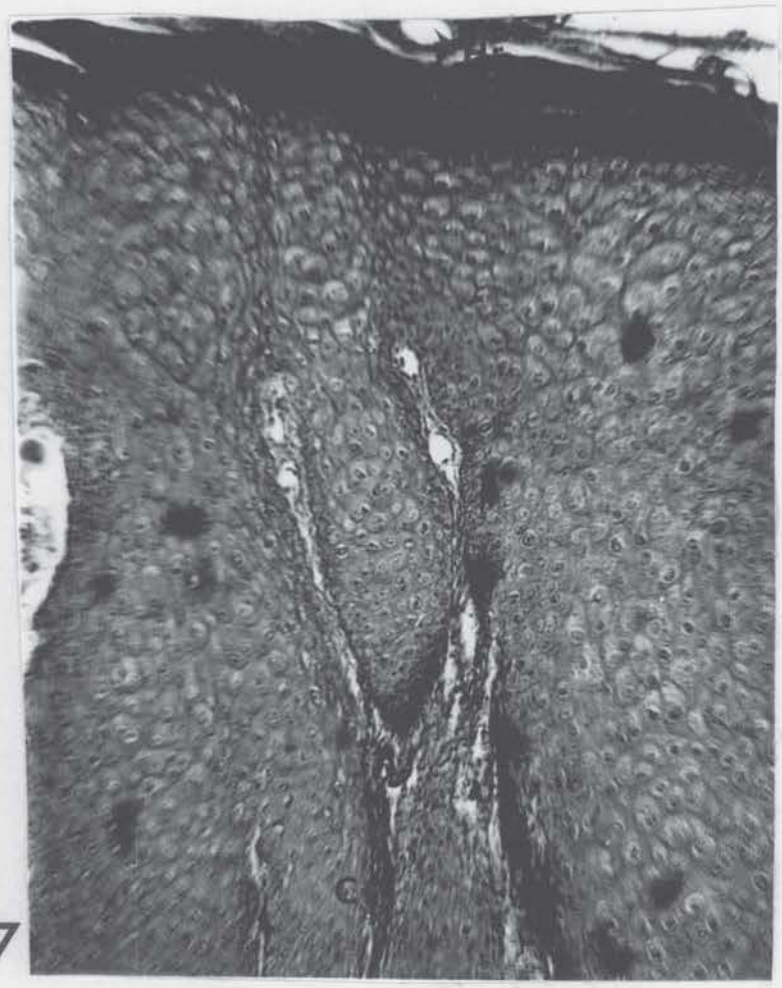

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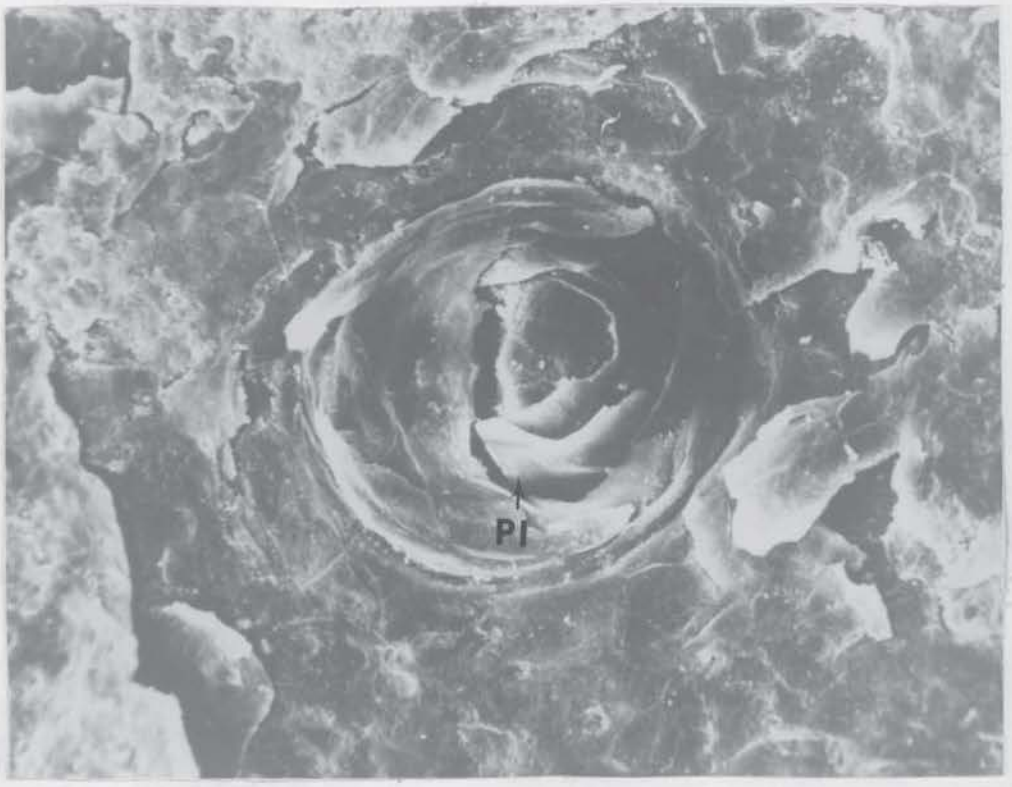

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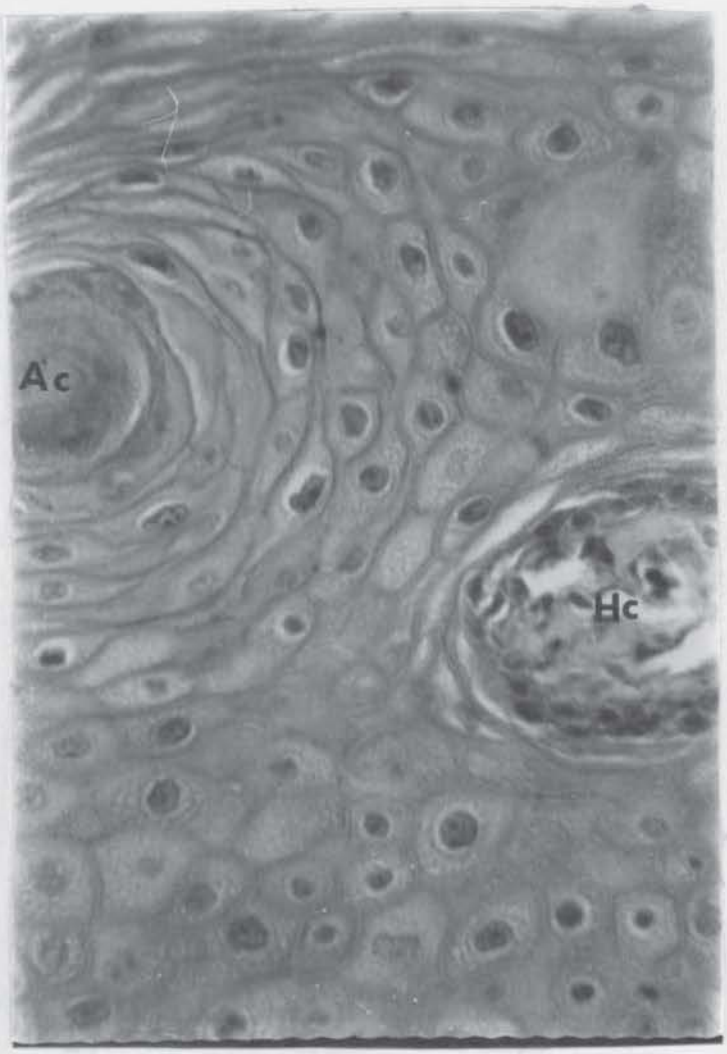

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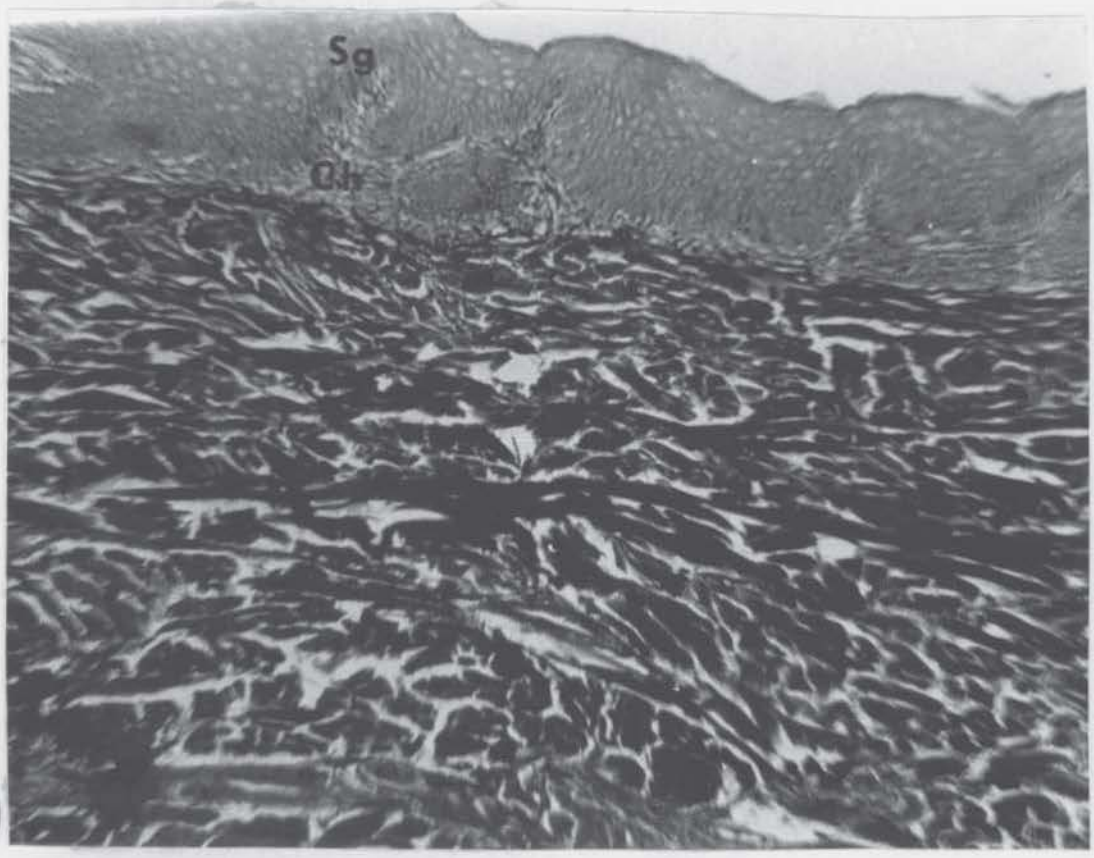

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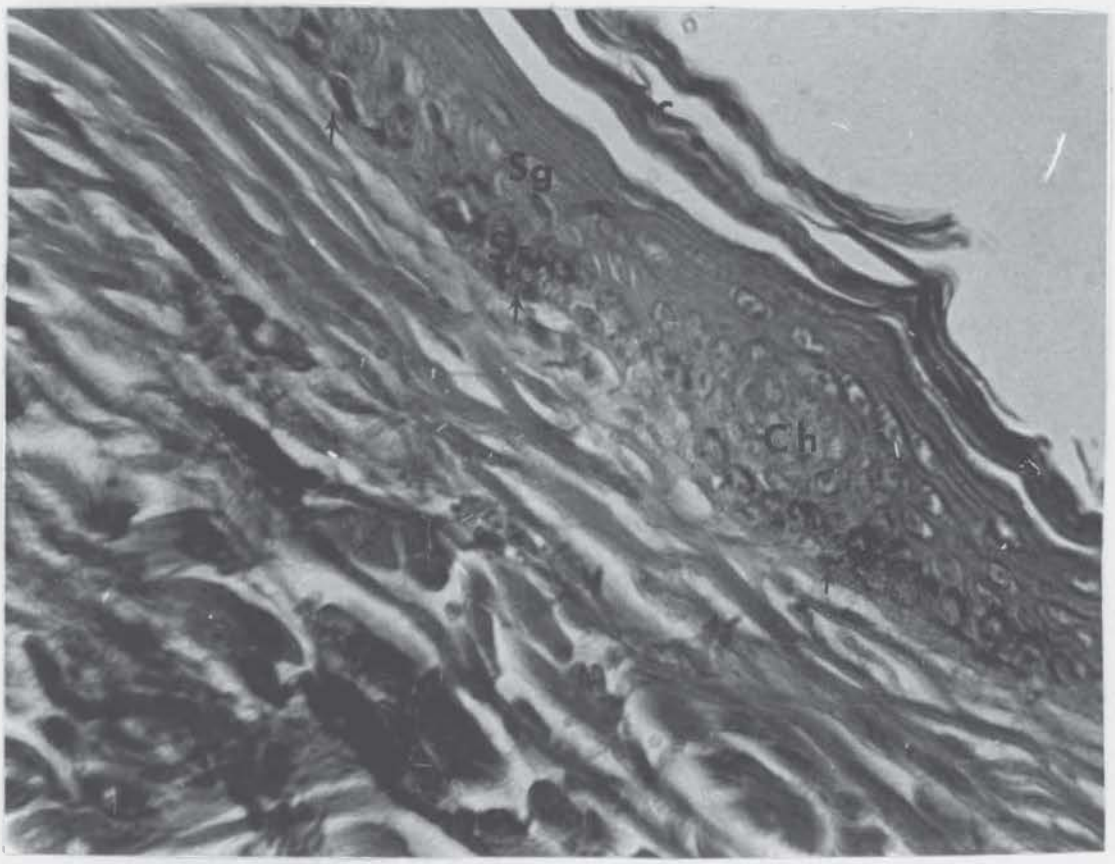

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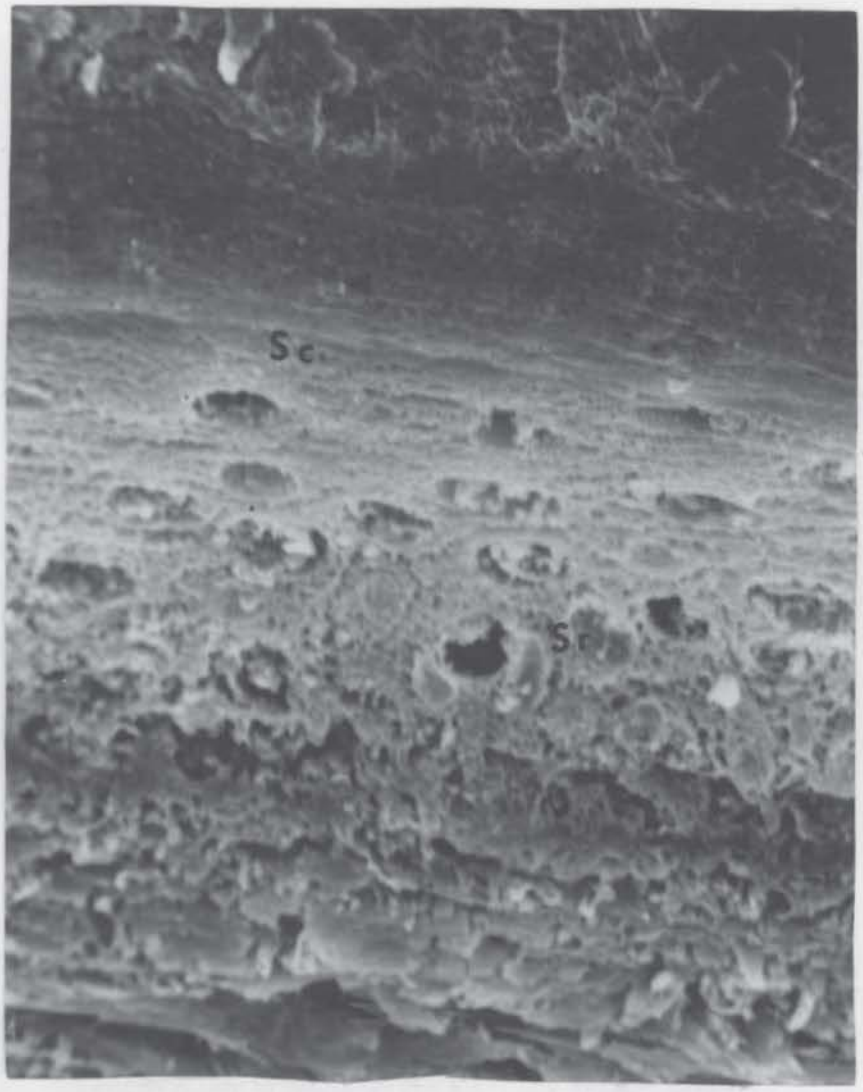

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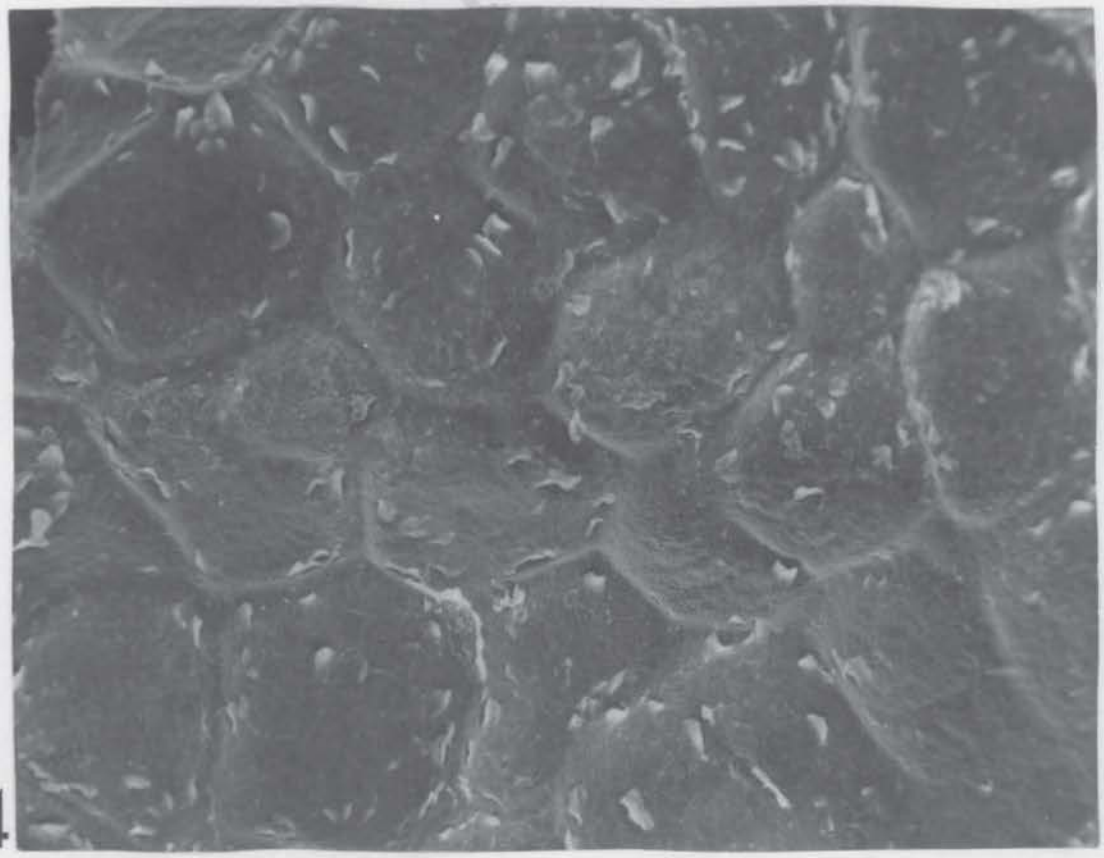

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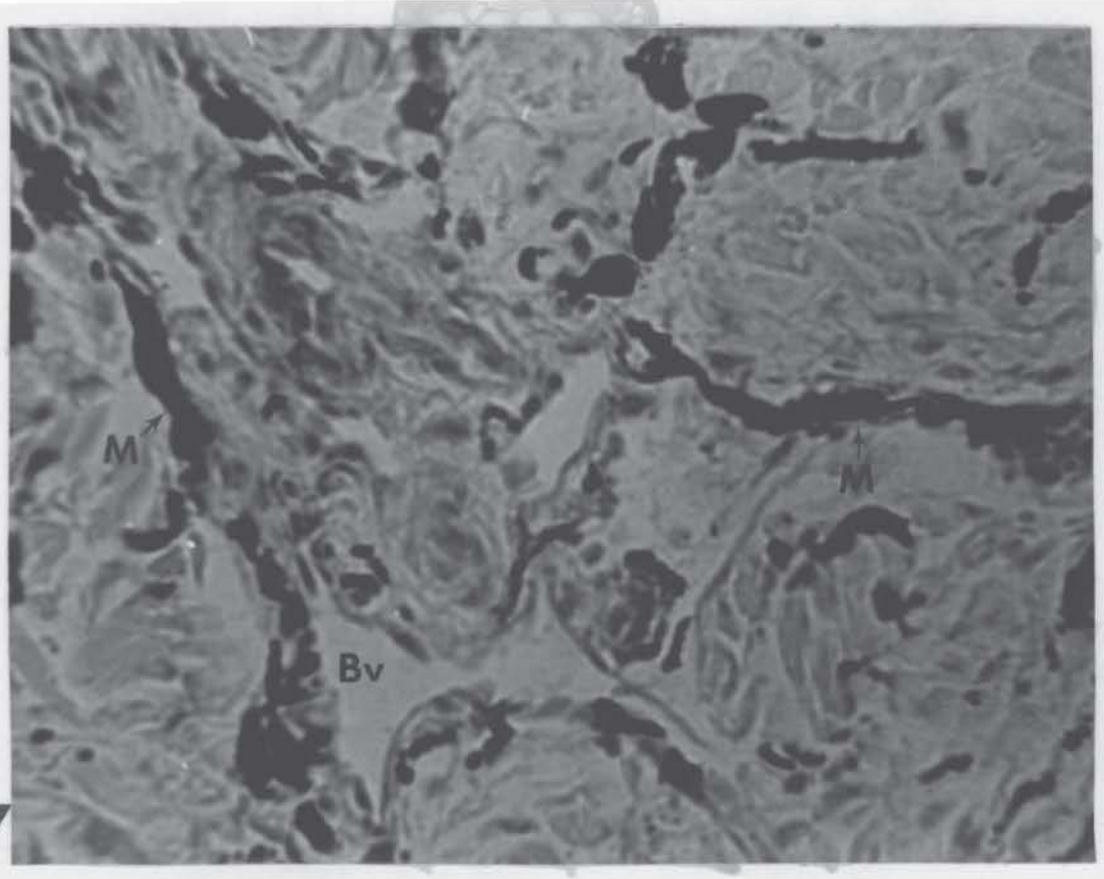

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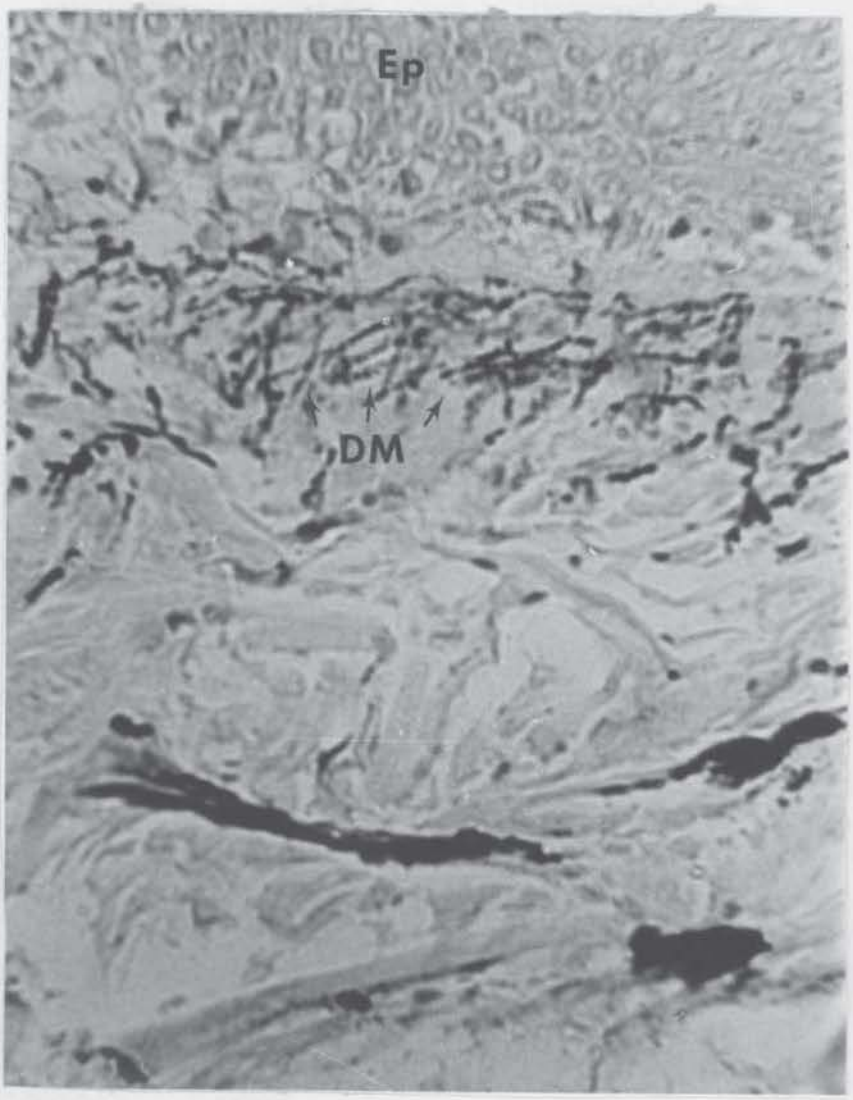

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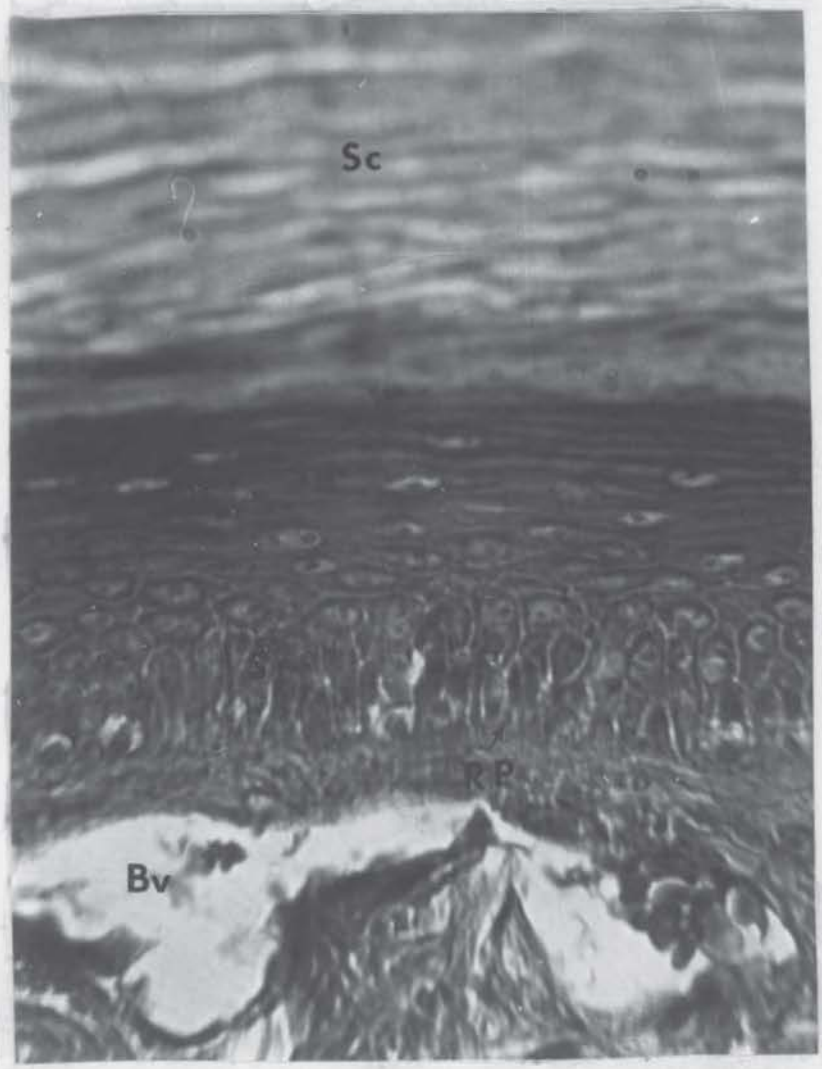

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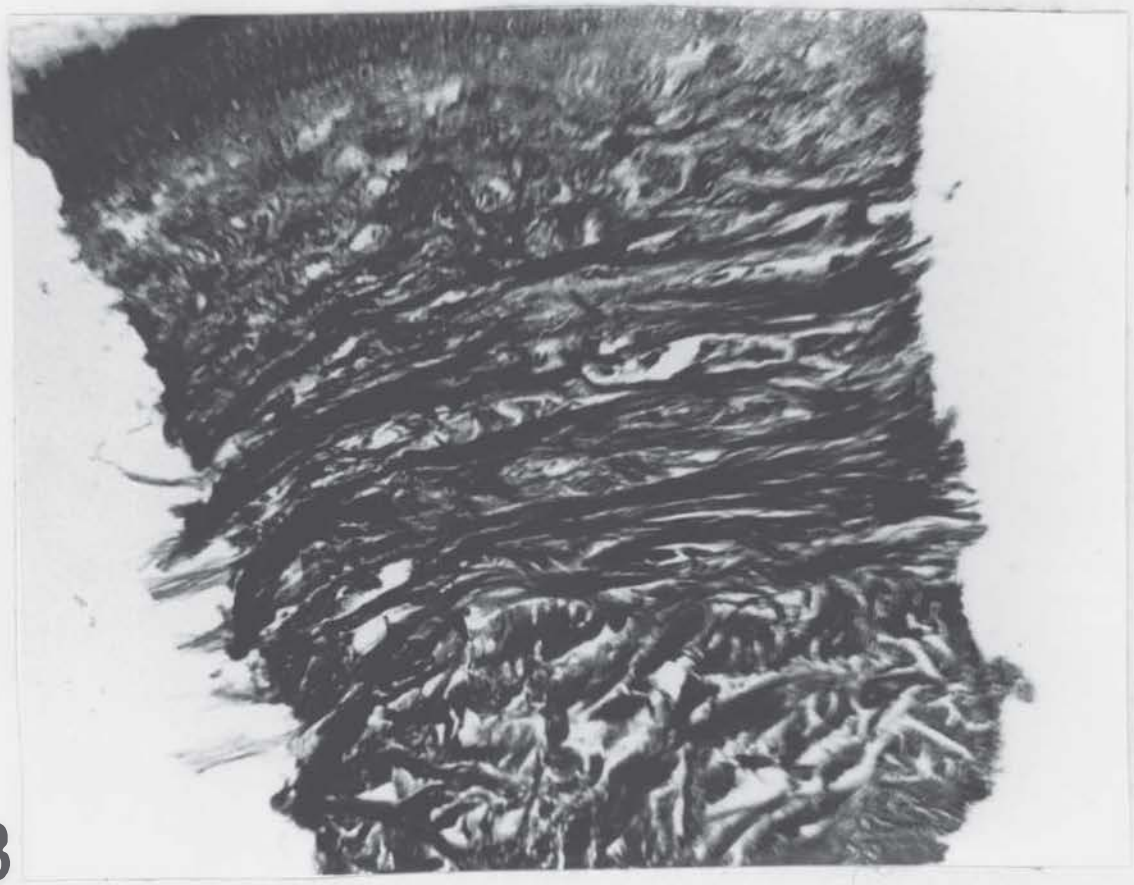

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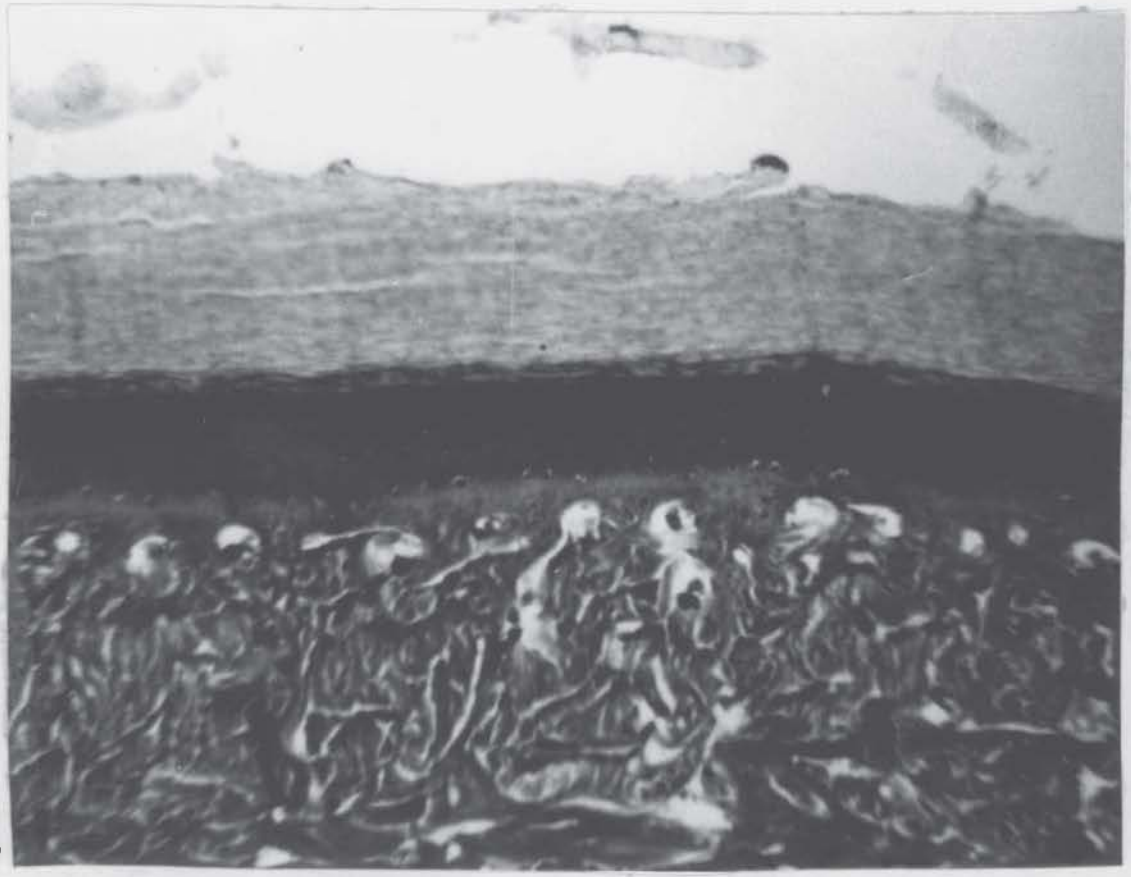

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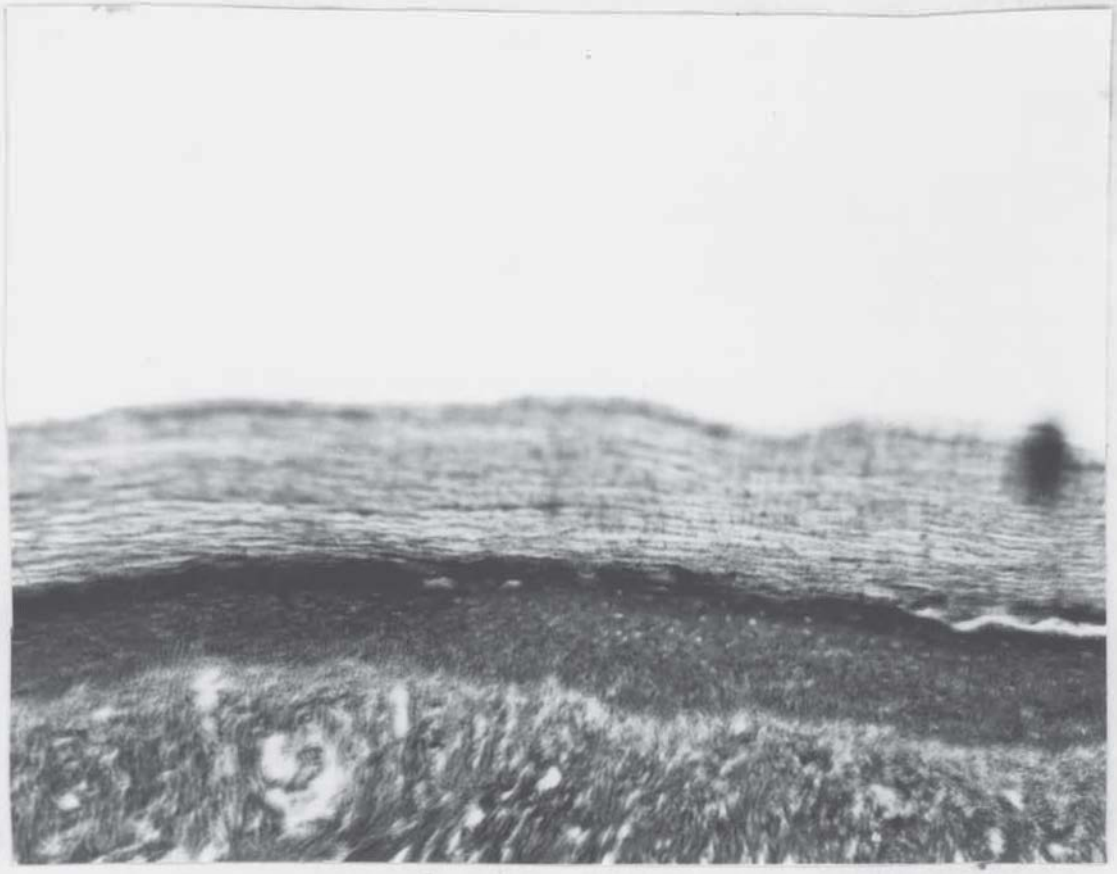

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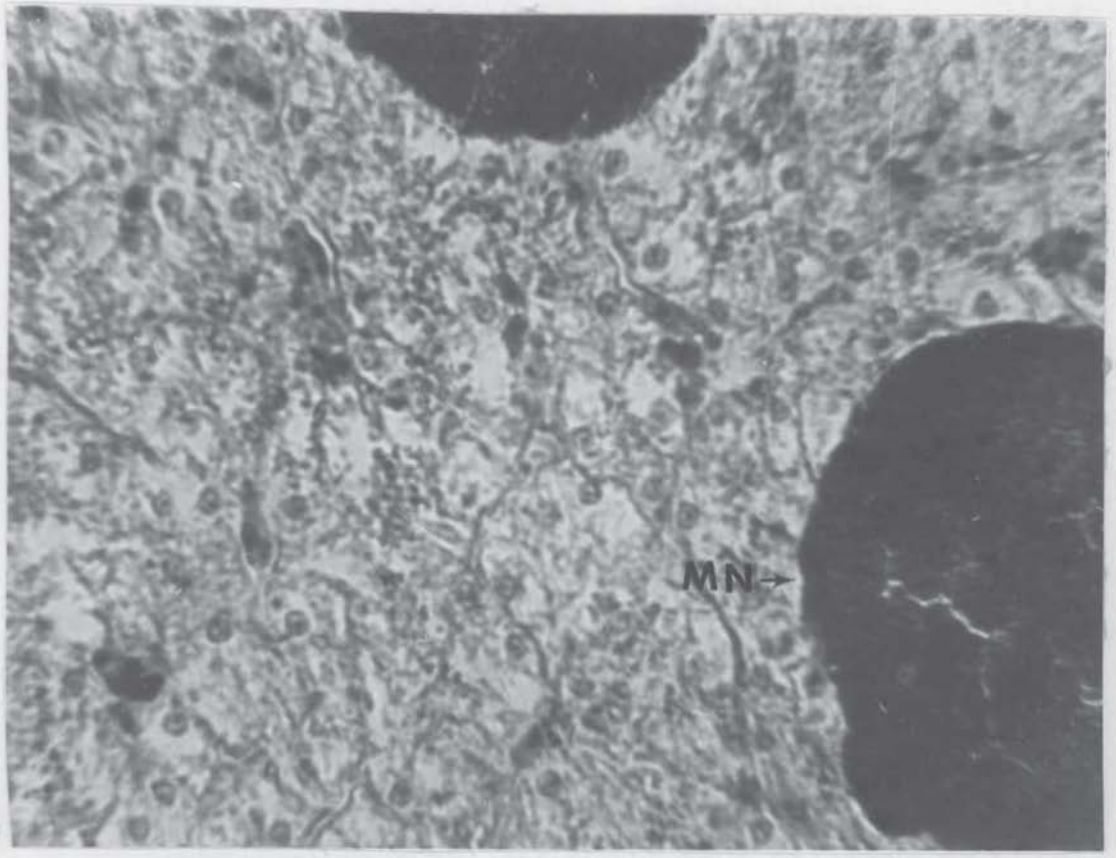

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